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Quantitative Study of the Structural Requirements of Phthalazine/Quinazoline Derivatives for Interaction with Human Liver Aldehyde Oxidase

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Aldehyde oxidase is a molybdenum-containing enzyme distributed throughout the animal kingdom. Although this enzyme is capable of metabolizing a wide range of aldehydes and *N*-heterocyclic compounds, there is no reported detailed study of physicochemical requirements of the enzyme-substrate interactions. The aim of this study, therefore, was to investigate quantitatively the relationships between the kinetic constants of aldehyde oxidase-catalyzed oxidation of some phthalazine and quinazoline derivatives (as substrates) and their structural parameters. Multiple regression and stepwise regression analyses showed that polarity of phthalazines (expressed as dipole moment μ, cohesive energy density δ_T and an indicator variable for hydrogen-bond acceptor ability of R1 substituent, HBA) had a negative effect on the enzyme activity (leading to the reduction of V_{max} and increase of K_m). Electron withdrawing substituents in the quinazoline series are favorable for interaction with the enzyme. This finding and also the relationships of $1/K_m$ of phthalazines with the energy of the lowest unoccupied molecular orbital and $\log V_{max}/\log K_m$ of phthalazines with degree of bonding of the two nitrogen atoms in the molecules are consistent with the mechanism of action. The reaction involves a nucleophilic attack on an electron-deficient sp^2-hybridized carbon atom and formation of an epoxide intermediate following the disruption of the aromatic structure.

Key words QSAR; aldehyde oxidase; phthalazine; quinazoline; enzyme-substrate interaction

Aldehyde oxidase [aldehyde: O_2 oxidoreductase EC 1.2.3.1] is a large dimeric protein with a molecular weight of approximately 300000 Da, composed of two identical subunits (150000 Da).[1,2] Each subunit contains 1 atom of molybdenum, 1 molecule of enzyme-bound flavin adenine dinucleotide (FAD), 4 atoms of non-heme iron and 4 atoms of acid-labile sulfur.[1,2] It can oxidase a wide range of substrates including aldehydes and *N*-heterocyclic compounds. The reaction catalyzed by this enzyme can be represented by the general equation:

$$RH + H_2O \rightarrow ROH + 2e^- + 2H^+$$

where RH and ROH are the substrate and the hydroxylated metabolite, respectively.[1,3] The reaction involves a nucleophilic attack on an electron-deficient sp^2-hybridized carbon atom which may belong to aromatic azaheterocycles containing the –CH=N– moiety (such as phthalazine and purine), aromatic or nonaromatic charged azaheterocycles containing the $-CH=N^+<$ moiety (*e.g.*, N^1-methylnicotinamide and *N*-methylphthalazinium) or aldehydes containing the –CH=O moiety (such as benzaldehyde and vanillin).[4] Aldehyde oxidase is widely distributed throughout the animal kingdom and many studies have been carried out using the enzyme prepared from various species. As this cytosolic enzyme is involved in the oxidation of the important compounds famciclovir,[5] methotrexate,[6] azathioprine,[7] quinine,[8,9] quinidine,[9] carbazeran,[10] allopurinol,[11] pyridoxal,[12] and N^1-methylnicotinamide,[12] an understanding of the manner of the interactions of aldehyde oxidase with its substrates would be useful in the design of efficient substrates or potent inhibitors for this enzyme. However, there is no report on the detailed study of the physicochemical requirements of aldehyde oxidase-substrate interactions. In order to obtain a better understanding of these interactions at the molecular level, in the present study, structure–activity relationships of some *N*-heterocyclic substrates of aldehyde oxidase including phthalazine and quinazoline derivatives were investigated quantitatively. The reaction schemes of phthalazine and quinazoline to the corresponding oxidation products are shown in Chart 1.

Experimental

The kinetic constants (K_m and V_{max}) were available for 14 phthalazine and 15 quinazoline derivatives (Tables 1 and 2).[13] The values have been measured by Beedham *et al.* using partially purified human liver enzyme fractions.[13] K_m, V_{max}, the ratio of V_{max}/K_m, $1/K_m$, and also the logarithm of these parameters were used as the biological data in the stepwise regression analyses against the structural parameters of the aldehyde oxidase substrates. The structural parameters used in this study are all calculated rather than experimentally derived parameters. These parameters are easily obtainable even for complex molecules and they can predict activity of compounds *a priori*. The structures were optimized using both the COSMIC force field[14] and, for quantum mechanical parameters, the AM1 semiempirical method. Structural parameters were generated mainly by MOPAC 7.0 for PC and also by molecular mechanical methods. The parameters were atomic charges on certain atoms in the main structure, the most positive and the most negative atomic charges on the substituents, molecular weight, energies of the highest occupied and the lowest unoccupied molecular orbitals, solvent accessible surface area calculated using the probe of 1.4 Å radius on the van der Waals surface of molecules, the highest and the lowest electrostatic potentials on

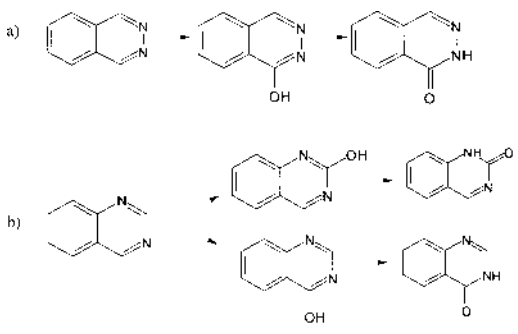

Chart 1. The Oxidation of Phthalazine (a) and Quinazoline (b)

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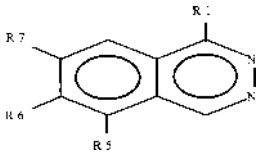
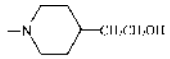
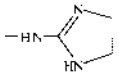
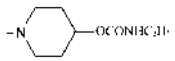
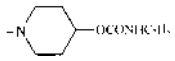
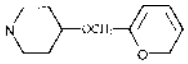
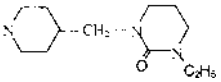
Table 1. Phthalazine Derivatives Used in QSAR Equations and the Corresponding K_m (μM) and V_{max} (μM/min/mg) Values



Phthalazine No.	R1	R5	R6	R7	K_m [a)]	V_{max} [a)]
P1	H	H	H	H	41	0.107
P2	Cl	H	H	H	9	0.068
P3	Cl	H	$-OCH_3$	$-OCH_3$	30	0.064
P4	$-OC_2H_5$	H	H	H	43	0.081
P5	$-C_6H_5$	H	H	H	9	0.047
P6	$-OC_6H_5$	H	H	H	150	0.018
P7		H	H	H	39	0.019
P8		H	H	H	35	0.006
P9		H	H	H	26	0.004
P10		H	$-OCH_3$	$-OCH_3$	52	0.060
P11		$-OCH_3$	$-OCH_3$	$-OCH_3$	130	0.019
P12		H	$-OCH_3$	$-OCH_3$	23	0.029
P13		H	$-OCH_3$	$-OCH_3$	220	0.033
P14	$NHCH_2CH_2N(CH_3)_2$	H	H	H	37	0.004

a) Data taken from ref. 13.

the solvent accessible surface of the molecule, dipole moments (calculated by the semiempirical AM1 method and also from charge-2 method), maximum interatomic distances between the substituents and the carbon atoms to which these are substituted, relevant degrees of bonding, and also indicator variables for hydrogen bonding donor and acceptor ability in the substituents. For phthalazines, molar volume, heat of vaporization and cohesive energy density were also calculated using the group contributions of Fedors.[15] The phthalazine and quinazoline derivatives were studied separately. In addition, the whole molecule parameters were extracted and employed to analyze the two groups of compounds together. Multiple regression and stepwise regression analyses were performed using MINITAB statistical analysis software.

Results and Discussion

Phthalazine Derivatives Table 3 contains the values of the structural parameters which were significantly correlated with the biological data of phthalazines. The following equation resulted from the stepwise regression analysis with the $1/K_m$ as the dependent variable:

$$1/K_m = -0.133\mathrm{HBA} - 0.36\mathrm{qR1} - 0.019\mu - 0.111E_{LUMO} + 0.066 \quad (1)$$
$$n=14, \quad r=0.925, \quad s=0.016$$

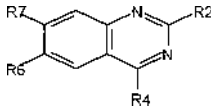
where HBA is the indicator variable for hydrogen bonding acceptor ability of the R1 substituent, qR1 is the atomic charge on the first atom of R1 (calculated by the AM1 method), μ is dipole moment calculated by the AM1 semiempirical method, and E_{LUMO} is the energy of the lowest unoccupied molecular orbital (the AM1 method). The numbers of descriptors used in Eq. 1 is rather large for the 14 observations, but the *t*-ratios of the variables proved that they were all statistically significant (the lowest *t*-ratio was 2.1 for E_{LUMO} ($p<0.05$)).

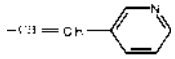
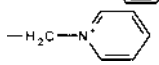
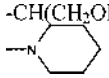
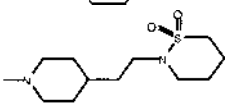
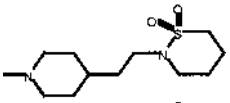
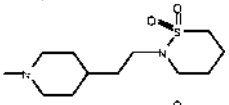
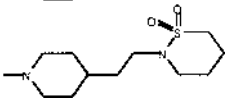
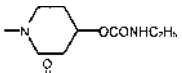
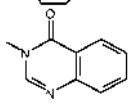
Equation 1 shows clearly that polarity of compounds increases the K_m; this can be concluded from the negative coefficients of both HBA and μ. The relationship with E_{LUMO} is in agreement with the assumed mechanism of oxidation, which involves a nucleophilic attack of the enzyme on the molecule. Using V_{max} and $\log V_{max}/\log K_m$ as the dependent variables in the stepwise regression analyses led to Eqs. 2 and 3.

$$V_{max} = -0.101E_{HOMO} - 0.0137\delta_T - 0.702 \quad (2)$$
$$n=14, \quad r=0.902, \quad s=0.015$$

In this equation E_{HOMO} is the energy of the highest occupied molecular orbital and δ_T is cohesive energy density calculated for the whole molecule. The higher E_{HOMO} values show the easier availability of the electrons in the highest occupied molecular orbital. Because the aromatic system is undergoing a nucleophilic attack, the lower E_{HOMO} will be desirable. δ_T can be considered as a polarity parameter, which has a negative effect on V_{max}.

Table 2. Quinazoline Derivatives Used in QSAR Equations and the Corresponding K_m (μM) and V_{max} (μM/min/mg) Values



No.	R2	R4	R6	R7	K_m [a)]	V_{max} [a)]
Q1	H	H	H	H	21	0.151
Q2	H	H	NH_2	H	41	0.080
Q3	H	H	NO_2	H	77	0.008
Q4	H	H	H	NH_2	140	0.009
Q5	CH_3	H	H	H	15	0.071
Q6		H	H	H	15	0.008
Q7		H	H	H	400	0.018
Q8	H	-CH(CH$_2$OH)$_2$	H	H	300	0.054
Q9	H		H	H	78	0.025
Q10	H		H	H	35	0.048
Q11	H		OCH_3	H	10	0.015
Q12	H		H	OCH_3	13	0.008
Q13	H		OCH_3	OCH_3	33	0.004
Q14	H		H	H	43	0.004
Q15	H		H	H	17	0.030

a) Data taken from ref. 13.

$$\log V_{max}/\log K_m = -18.4D_{NN} - 1.07qR6 + 23.07 \quad (3)$$
$$n=14, \quad r=0.904, \quad s=0.171$$

where D_{NN} is degree of bonding of the two nitrogen atoms in the molecules and qR6 is the atomic charge on the first atom of R6 (the AM1 method). The negative relationship with qR6 could be due to the better enzyme fitting of the compounds with $-OCH_3$ substituent (which can act as a possible hydrogen bond acceptor) in position 6 than those without a substituent. The negative slope for D_{NN} is expected because there is a bond breakage within the ring during the oxidation.

The relationships with $\log V_{max}$, $\log K_m$ and $\log(K_m/V_{max})$ are not as good as the above mentioned correlations. For example, $\log K_m$ has a poor correlation with HBA ($r=0.578$) and the following is the equation with $\log V_{max}$ which, again, expresses the negative effect of polarity on V_{max}:

$$\log V_{max} = 39.9qN2 - 0.31\mu + 8.58 \quad (4)$$
$$n=14, \quad r=0.897, \quad s=0.526$$

where μ has been calculated from the charge-2 method[16] and qN2 is atomic charge on the N2 nitrogen atom, near R1. The higher charges on the nitrogen atom indicate the greater electron withdrawing effect of the R1 substituent and consequently the higher suitability of the ring to be subjected to the nucleophilic attack.

Quinazoline Derivatives Considering the structures of the quinazolines (Table 2), it is clear that there are two possible oxidation sites in the molecules, namely, C2 and C4 carbon atoms. In Q5—Q7 the C2 and in Q8—Q15 the C4 carbons are substituted and are no longer available for oxidation. This means that the substrate binding to the enzyme is performed in different manners for the two subsets. In the case of Q1—Q4, with no substituent in any of the C2 or C4 positions, the decision cannot be made as to which class they belong. Therefore, quinazolines with R4 substituent (Q8—Q15) were first analyzed separately. The following equations were obtained for the Q8—Q15:

$$(V_{max}/K_m)\cdot 1000 = 0.0672sESP^+ + 39.3q_4 - 10.8 \quad (5)$$
$$n=7, \quad r=0.887, \quad s=0.398$$

$$K_m = -3.16sESP^+ + 107 \quad (6)$$
$$n=6, \quad r=0.820, \quad s=14.75$$

$$\log K_m = -0.0372sESP^+ - 0.364HBA_{6,7} + 2.293 \quad (7)$$
$$n=6, \quad r=0.945, \quad s=0.241$$

Table 3. Structural Parameters for Phthalazine Derivatives Used in the QSAR Equations

No.	E_{HOMO}	E_{LUMO}	μ (AM1)	D_{NN}	qR1	qR6	qN2	δ_T	μ (Charge-2)	HBA
P1	−9.553	−0.903	4.570	1.282	0.169	0.139	−0.240	12.719	5.08	0
P2	−9.573	−1.149	4.999	1.303	0.036	0.143	−0.230	14.686	5.32	0
P3	−9.348	−1.117	6.362	1.310	0.039	−0.209	−0.240	13.301	6.03	0
P4	−9.110	−0.710	3.216	1.292	−0.203	0.138	−0.264	11.984	4.10	1
P5	−9.192	−0.992	4.308	1.319	−0.044	0.140	−0.247	12.500	4.98	0
P6	−9.053	−0.833	3.280	1.295	−0.161	0.140	−0.263	12.553	4.31	1
P7	−8.885	−0.756	5.128	1.313	−0.221	0.137	−0.270	12.958	6.33	1
P8	−8.608	−0.839	4.515	1.337	−0.252	0.140	−0.290	13.626	7.17	1
P9	−8.898	−0.903	4.186	1.338	−0.217	0.141	−0.288	13.895	8.63	1
P10	−9.117	−0.997	5.461	1.315	−0.215	−0.211	−0.272	12.200	4.69	1
P11	−8.859	−0.903	5.696	1.315	−0.214	−0.216	−0.270	12.080	4.41	1
P12	−8.592	−0.893	4.041	1.312	−0.230	−0.211	−0.273	11.154	4.60	1
P13	−8.864	−0.793	5.270	1.314	−0.241	−0.212	−0.268	11.752	4.43	1
P14	−8.614	−0.733	4.070	1.322	−0.262	0.139	−0.294	11.642	5.41	1

The AM1 derived parameters are E_{HOMO} and E_{LUMO}, the highest occupied and the lowest unoccupied molecular orbital energies, respectively, μ (AM1), dipole moment, D_{NN}, degree of bonding of the two nitrogen atoms, qR1 and qR6, the atomic charges on the first atoms of R1 and R6, respectively. HBA is hydrogen bonding acceptor indicator variable for R1, and qN2 is the atomic charge on the N2 nitrogen atom calculated by the charge-2 method.

Table 4. Structural Parameters for Quinazoline Derivatives

No.	q_4	$sESP^+$	$sESP^-$	q_{H2}	$R2R4^+$	maxR	D_{23}	$HBA_{6,7}$
Q1	0.123	23.952	3.967	0.1921	0.108	1.110	1.1963	0
Q2	0.112	19.424	0.253	0.1926	0.101	1.109	1.1968	1
Q3	0.124	30.669	8.785	0.2011	0.101	1.115	1.1978	1
Q4	0.116	19.911	−0.963	0.1893	0.101	1.110	1.1995	1
Q5	0.123	23.582	−16.506	—	0.100	2.176	1.1722	0
Q6	0.119	22.738	−27.96	—	0.100	6.442	1.1710	0
Q7	0.123	162.583	62.441	—	0.638	6.079	1.1948	0
Q8	0.147	46.941	−38.476	0.1923	0.329	3.483	1.2152	0
Q9	0.260	12.810	−12.914	0.1893	0.108	5.334	1.2270	0
Q10	0.260	24.627	−32.151	0.1897	0.108	11.502	1.2321	0
Q11	0.254	25.279	−31.331	0.1923	0.108	11.572	1.2297	1
Q12	0.255	25.172	−31.874	0.1913	0.108	11.485	1.2199	1
Q13	0.250	20.038	−36.613	0.1916	0.108	11.510	1.2364	1
Q14	0.236	23.519	−37.381	0.1912	0.205	9.3128	1.2256	0
Q15	0.267	32.956	−36.233	0.1974	0.108	7.5413	1.2207	0

q_4 is the atomic charge on C4, $sESP^+$ and $sESP^-$ are the highest and the lowest electrostatic potentials on the solvent accessible surface of the R4 or R2 substituents, q_{H2} is the atomic charge on the hydrogen atom connected to C2 (for the derivatives without an R2 substituent), $R2R4^+$ is the highest atomic charge on the R2 or R4 substituents, maxR is the maximum interatomic distance between R4 substituent and the carbon atom to which it is connected, D_{23} is the bonding order between C2 and N3 atoms, and $HBA_{6,7}$ is the indicator variable for the hydrogen bonding basicity in R6 or R7 substituents.

in which $sESP^+$ is the highest electrostatic potential on the solvent accessible surface of the substituent R4, q_4 is the atomic charge on C4, and $HBA_{6,7}$ is the indicator variable for the presence of hydrogen bond acceptor ability in R6 or R7 substituents. The *t*-values for $sESP^+$ and q_4 in Eq. 5 and $sESP^+$ and $HBA_{6,7}$ in equation 7 are 3.41 ($p<0.019$), 4.03 ($p<0.010$), 4.38 ($p<0.012$) and 3.75 ($p<0.020$), respectively, which show that despite the rather high ratio of structural parameters per observations used in these equations, the independent variables are statistically significant. Q8 was an outlier and was consequently deleted from Eqs. 6 and 7. Equations 5—7 show that the higher electrostatic potential on the R4 increases the enzyme activity. This could be due to the higher electron withdrawing ability of the substituents with high $sESP^+$, which leads to the more nucleophilic C2 carbon, which is necessary for the oxidation process. Q8 is an exception to this explanation, as, despite the high $sESP^+$ value it has a high K_m value. The positive coefficient of q_4 in Eq. 5, which is also due to the electron withdrawing nature of the R4, is in accordance with this explanation. This was also observed for the phthalazine series in which the R1 substituent was required to be electron withdrawing. However, another explanation for the desirability of high $sESP^+$ in substrates could be the better enzyme binding of such compounds through dipole–dipole interactions.

$HBA_{6,7}$ has a negative coefficient in Eq. 7 which indicates the better affinity of the enzyme to the substituents with H-bonding acceptor ability. This result is in agreement with the finding about phthalazine derivatives, which showed that phthalazines with methoxyl substituent in a similar position (R6 and R7 in Table 1) were better substrates of aldehyde oxidase (Eq. 3). It is worthwhile to mention that addition of $HBA_{6,7}$ as the second parameter to Eq. 6 increases the regression coefficient to $r=0.991$.

When the four quinazoline derivatives without R2 or R4 substituents (Q1—Q4) were incorporated in the analyses, although similar correlations with $sESP^+$ still existed, the other parameters worked better:

$$K_m = -4.15 sESP^+ - 7.16 q_{H2} + 899 \quad (8)$$
$$n=11, \quad r=0.725, \quad s=29.8$$

In the final stage, correlations, which could cover all the

quinazolines were sought. To this end, some of the parameters needed to be redefined, for example, $sESP^+$ and $sESP^-$ are, respectively, the highest and the lowest electrostatic potentials on the solvent accessible surface of the substituent R4, or R2 (where there is no R4 substituent). The following are informative correlations:

$$\log K_m = 0.0125 sESP^- + 1.73 \quad (9)$$
$$n=14, \quad r=0.764, \quad s=0.302$$

$$\log K_m = 2.54 R2R4^+ - 0.086 maxR + 13 D_{23} - 14.0 \quad (10)$$
$$n=15, \quad r=0.892, \quad s=0.252$$

Q8 was an outlier from the correlation between $\log K_m$ and is excluded from Eq. 9. In Eq. 10, $R2R4^+$ is the most positive atomic charge (calculated by the charge-2 method) in R4 (or R2 for Q5-Q7), maxR is the AM1 calculated maximum interatomic distances between R4 substituent and the carbon atom to which it is substituted (in the case of Q5—Q7, the maximum distance between R2 substituent and C2 carbon is intended), D_{23} is the bonding order between C2 and N3 atoms calculated by AM1. It can be seen in Table 4 that the highest $R2R4^+$ value belongs to Q7, Q8, and Q14. These are the compounds with hydrogen bonding donor ability, which, presumably, is not favorable for enzyme binding (probably because of the low lipophilicity of such compounds). The coefficient of maxR indicates that for this set of substrates the longer chains are preferred. The relationship with the D_{23} is in accordance with the mechanism of oxidation in which the disruption of the aromaticity occurs; the smaller bond order means that bond breaking requires less energy.

Equation 9 shows that the lower $sESP^-$ values result in lower K_m values and better enzyme affinity. This could be a result of stronger dipole–dipole interactions of such substituents (which have strong electronegative atoms) with the active site of the enzyme. This reasoning could be correct only if it is assumed that the R2 and R4 substituents of quinazolines occupy the same place in the enzyme active site. Another explanation, which does not need this assumption, is the electron withdrawing effect of the electronegative atoms in the substituents, which decrease the resonance electron donating property of the nitrogen atom of R4 in structures like Q9—Q15.

Phthalazine and Quinazoline Derivatives In order to derive correlations for the combination of the R2 and R4 substituted quinazolines and also phthalazines (29 compounds), it was assumed that the R2 substituent of quinazoline numbers 5, 6, and 7, the R4 substituent of the rest of the quinazolines (Table 2) and the R1 substituent of the phthalazines (Table 1) occupy the same place in the enzyme active site; this was only necessary for substituent parameters. Attempts to generate equations covering both groups of compounds were not successful. Aldehyde oxidase is able to oxidize a wide range of substrates ranging from small molecules such as pyridoxal[12] to large and complex molecules like methotrexate.[6] To date, the detailed characteristics of the active/binding site of this enzyme have not been fully understood. However, it seems that the active/binding site of aldehyde oxidase has a marked flexibility allowing this enzyme metabolize diverse compounds. Furthermore, different isozymes have been found for aldehyde oxidase from different sources.[13,17—19] Although, only one major isozyme has been reported in human liver fractions to date,[13] the possible existence of other isozymes of human liver cannot be excluded.

Conclusion

Based on the results obtained, electronegative atoms on R2 or R4 substituents of quinazolines (higher $sESP^+$ and lower $sESP^-$) are favored by the enzyme, as they reduce electron density on the c4 or c2, respectively. This relationship is consistent with the mechanism of aldehyde oxidase action, which involves a nucleophilic attack on an electron-deficient sp^2-hybridized carbon atom and consequently the disruption of the aromatic structure. This conclusion is also supported by the presence of q_4 in Eq. 5 and D_{23} in Eq. 10. For the phthalazine series the presence of qN in Eq. 4, E_{LUMO} in Eq. 1 and E_{HOMO} in Eq. 2 indicates a similar mechanism of action. There was a correlation with maxR (Eq. 10) which can be considered a steric parameter but this relationship may also be a result of better electronic requirements of the longer substituents and there is need for more evidence. According to the results obtained, the polarity (hydrophilicity) of the substrates expressed as dipole moment μ or cohesive energy density δ_T or HBA has a negative effect on the enzyme activity. The results of this study can also give clues about the structure of the binding site of the enzyme. It was assumed that R2 and R4 substituents of quinazolines and R1 substituent of phthalazines occupy the same region in the enzyme active site. Although we did not find strong evidence to prove this assumption, there was no evidence to prove it wrong either.

The most important parameters for phthalazines are hydrogen bonding basicity indicator variable (HBA) and the energy of HOMO, which are the first parameters to enter the regression equation in stepwise-regression analysis with $1/K_m$ and V_{max} (Eqs. 1 and 2). For the quinazoline series the highest electrostatic potential of the R4 or R2 substituents ($sESP^+$) appeared in most of the equations.

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